Comparison of Cytokine Content Between the First and Second Clot Stages of the Thrombinator™ Device

Arthrex Research and Development

Objective

This study compared the first and second clot stages of the Thrombinator device when using whole blood (WB) or platelet-poor plasma (PPP) derived from the ACP Max™ system. The differences in cytokine content between the two stages and the different fluids were evaluated.

Materials and Methods

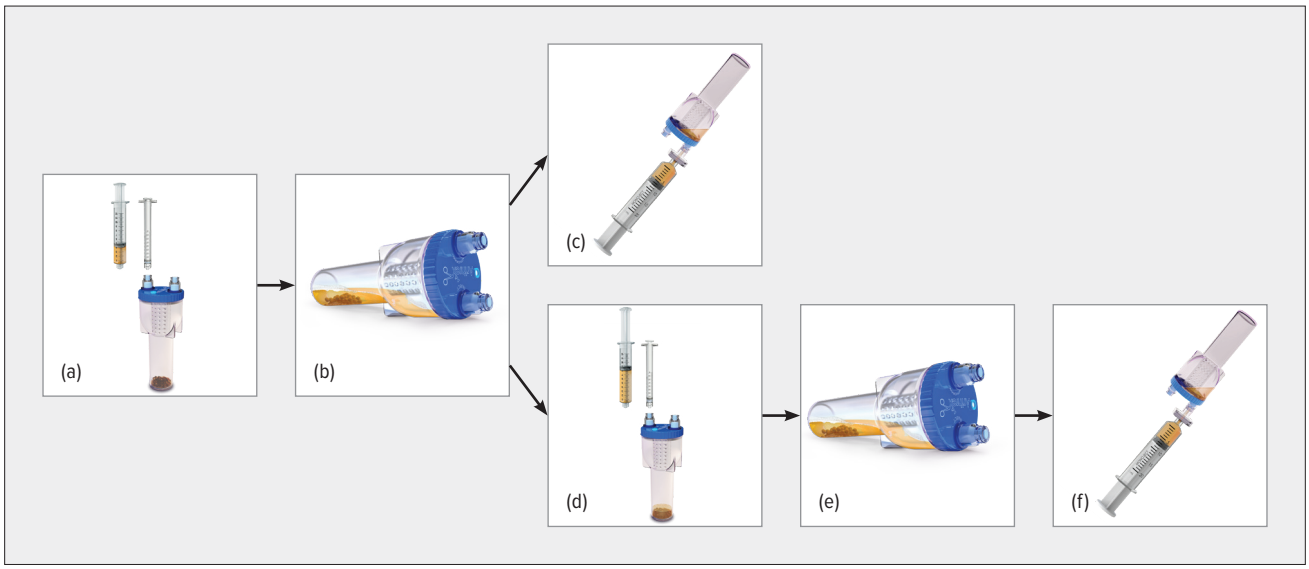
Blood Collection

A total of 105 mL of blood was collected from each of three donors (n = 3), using 13.3% citrate dextrose solution A (ACD-A) as the anticoagulant. Blood was drawn via standard arm venipuncture into two syringes preloaded with ACD-A. A small volume of anticoagulated blood from each donor was aliquoted for baseline complete blood count analyses. 20 mL of anticoagulated blood from each donor was moved to separate Thrombinator devices and centrifuged at 4000 rpm for 10 minutes at room temperature using a Hettich Rotofix centrifuge. The resulting plasma was collected and aliquoted for baseline circulating cytokine analysis.

PPP Collection

60 mL of anticoagulated blood from each donor was moved to individual ACP Max devices. The samples were spun at 3200 rpm for 6 minutes using a Hettich Rotofix centrifuge. The resulting PPP was collected into separate syringes until the PPP line was 4 mL above the red blood cell line.

Figure 1. Processing of WB or PPP from 1 donor with the Thrombinator devices. **(a)** Add 0.1 mL $CaCl_2$ and 4 mL PPP or WB to each of 2 Thrombinator devices. **(b)** Mix well and keep at room temperature until clots form. **(c)** Shake both devices and remove the fluid (clot 1) from one device. **(d)** Add 0.2 mL $CaCl_2$ and 8 mL PPP or WB to the remaining device. **(e)** Mix well and keep at room temperature until clot forms. **(f)** Shake and remove the fluid (clot 2) from the second device.



Thrombinator™ Device Processing

Following collection from each donor, PPP was processed using the Thrombinator devices (Figure 1). Two devices were used per donor per fluid group. One device was stopped after the first clot formation, while the other device continued the process until the second clot was formed.

First, 0.1 mL of 10% $CaCl_2$ was injected into each device, followed by 4 mL of either WB or PPP from each donor (Figure 1a). The contents were mixed thoroughly and left at room temperature on the benchtop to clot (Figure 1b). Once clotting occurred, all devices were shaken to break the clots. The serum from one device per donor and fluid group was then removed, filtered through the provided syringe filter, and designated as clot 1 (Figure 1c). Aliquots were collected and frozen at -80 °C. Next, 0.2 mL of 10% $CaCl_2$ was injected into the remaining Thrombinator devices, followed by 8 mL of the same fluid used in the first clot (Figure 1d). After thorough mixing, the devices were placed on the benchtop at room temperature to clot (Figure 1e). Once clotting had occurred, the devices were shaken to break the clots. The resulting serum was removed and filtered through the provided syringe filter, creating clot 2 (Figure 1f). Aliquots were collected and frozen at -80 °C.

Analysis

After collection, all samples were thawed and analyzed for cytokine content. Measurements were performed using the Human Cytokine A Premixed Magnetic Luminex Performance Assay (R&D Systems), excluding CCL5/RANTES, in accordance with the manufacturer's instructions. The assay was read using a Luminex MAGPIX (Diasorin). Statistical analysis was conducted using a one-way ANOVA, followed by a Holm-Sidak test for multiple comparisons when significant differences were detected ($\alpha = .05$).

Results

Figure 2 demonstrates the recovered volume and the fraction of the original volume for each group, categorized by clot and fluid type. Significant differences in recovered volume were observed between clot 1 and clot 2 for both the WB ($P = .013$) and PPP ($P = .008$) groups. However, a significant difference in the fraction of the original volume was only found between clots 1 and 2 in the PPP group ($P = .037$).

Figure 2. (a) Recovered volume and **(b)** fraction of the original volume for each group (average ± standard deviation; n = 3).

* Indicates statistically significant differences between clots 1 and 2 within the same fluid type.

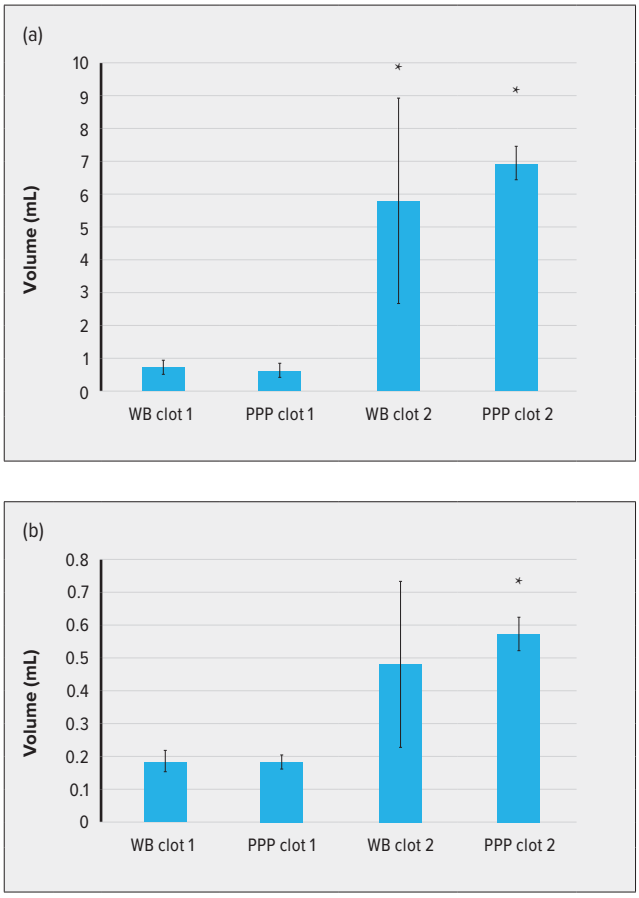
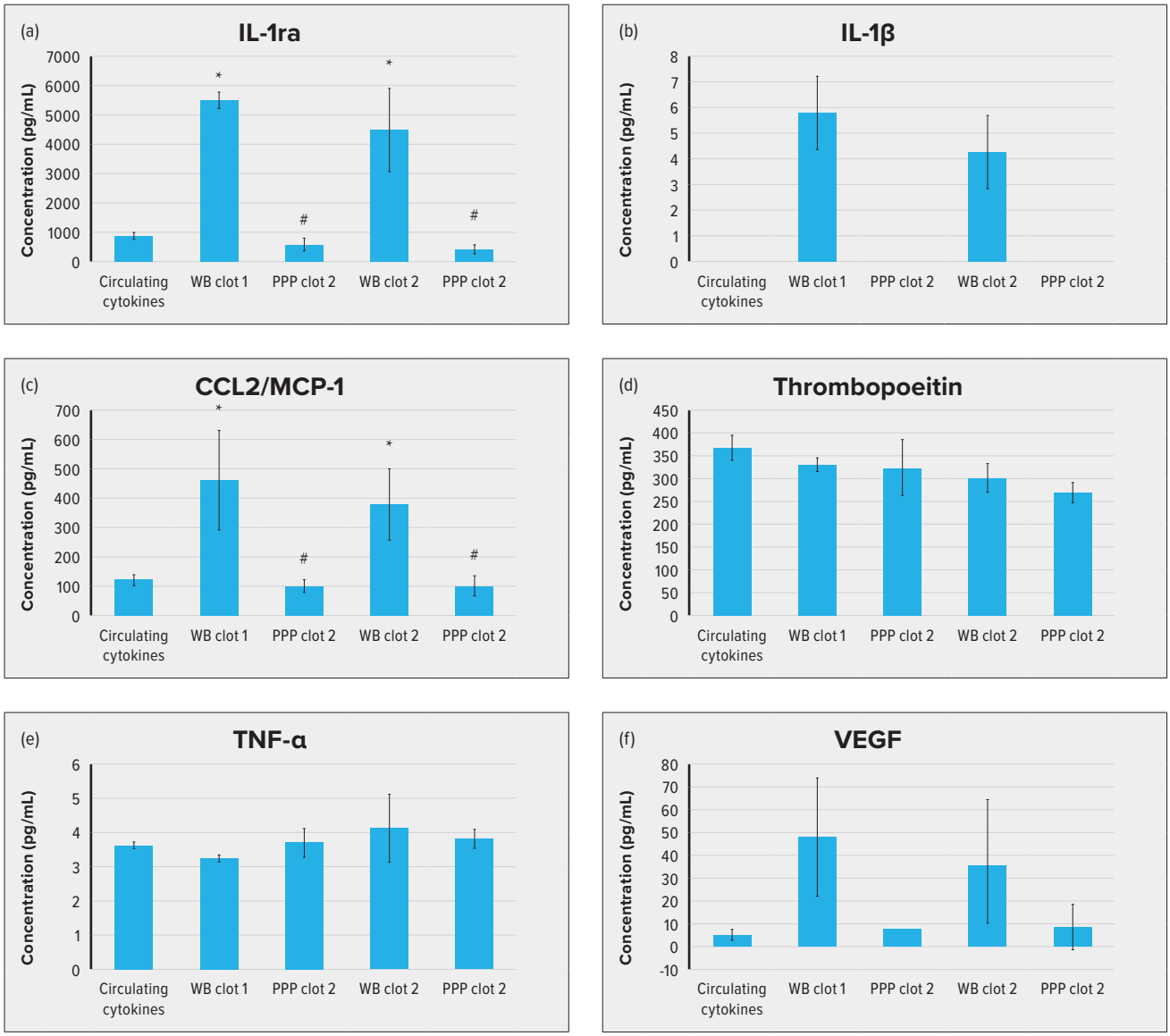


Figure 3 demonstrates the concentrations of various cytokines measured after each clot formation within each group. Cytokine levels were compared to baseline levels obtained immediately after blood collection and between WB and PPP within the same clot group. For IL-1ra (Figure 3a), both WB clots showed significant differences compared to circulating levels ($P < .001$). PPP clots were significantly lower than their WB counterparts ($P < .001$) but did not differ significantly from circulating levels. For IL-1β (Figure 3b), a similar trend was observed in both WB clots; however, cytokine levels in the circulating and PPP groups were below detection levels. CCL2/MCP-1 (Figure 3c) levels were significantly different in both WB clots compared to circulating levels ($P < .05$), while the PPP groups were significantly different from the WB groups ($P < .04$) but not from circulating levels. No significant differences were observed for thrombopoietin (Figure 3d) or TNF-α (Figure 3e). Although VEGF (Figure 3f) levels did not differ significantly, WB clots showed a trend toward higher concentrations.

Figure 3. Concentrations of **(a)** IL-1ra, **(b)** IL-1β, **(c)** CCL2/MCP-1, **(d)** thrombopoietin, **(e)** TNF-α, and **(f)** VEGF across all groups (average ± standard deviation; n = 3 for all groups except IL-1β where circulating cytokines = 0, PPP clot 1 = 0, and PPP clot 2 = 0).

* Indicates a significant difference from the circulating cytokine group.

# Indicates a significant difference between WB and PPP within the same clot group.



Discussion

The primary function of thrombin generation is to convert fibrinogen to fibrin. The Thrombinator™ device is comprised of synthetic borosilicate beads, which stimulate autologous fluid (PPP or whole blood) to produce thrombin in a two-stage process. The first step introduces autologous fluid to the system, which initiates clot formation and produces a prothrombinase complex.[1,2] The next step uses prothrombinase and additional autologous fluid to convert prothrombin into thrombin.[1,2] During these bioactive processes, cells and molecules can interact and cause a release of other biological factors beyond prothrombin and thrombin. These molecules consist of a variety of proteins that can be trapped within the final clot and eventually be released into the surrounding tissue. This experiment sought to determine the cytokine concentrations of the serum produced by the Thrombinator device after each clotting stage using either WB or PPP.

The results of the volume test suggest that a smaller proportion of fluid becomes trapped in the clot during the second stage compared to the first. Cytokine levels were also measured at each stage in both the whole blood and PPP groups. IL-1ra, an anti-inflammatory cytokine that competitively inhibits IL-1β,[3] was significantly elevated in WB groups compared to both baseline and PPP. This is likely due to the presence of white blood cells (WBCs) in WB, which are largely absent in PPP. WBCs trapped within the clot may secrete IL-1ra. Similarly, IL-1β, a potent pro-inflammatory cytokine,[4] was increased in both WB clots compared to baseline and PPP. However, the IL-1β assay was limited by sample concentrations that fell below the lowest value on the standard curve, resulting in unreadably low values. CCL2/MCP-1 acts as a chemokine, attracting monocytes, memory T cells, and dendritic cells to sites of inflammation.[5,6] This cytokine was significantly increased in WB over baseline and PPP. Thrombopoietin, a key regulator of platelet production,[7] was decreased in all groups compared to baseline, though not significantly. This may suggest high levels of platelet activation and subsequent thrombopoietin breakdown, as thrombopoietin is directly regulated by platelets.[7] TNF-α, a pro-inflammatory cytokine, showed no significant differences between groups. Lastly, VEGF, a growth factor involved in angiogenesis, showed no significant differences, although WB groups tended to have higher concentrations than both baseline and PPP. Overall, these findings suggest that the autologous serum generated by the Thrombinator device contains not only thrombin but also a range of cytokines and growth factors that may contribute to healing.

References

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